Brief Definitive Report

TRIGGERING T CELLS BY OTHERWISE INERT HYBRID ANTI-CD3/ANTITUMOR ANTIBODIES REQUIRES ENCOUNTER WITH THE SPECIFIC TARGET CELL

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Under normal circumstances, T cells are triggered when the TCR binds to the MHC antigen complex on another cell. This activation can also be induced by antibodies (Abs) that bridge the CD3/TCR complex to a structure on another cell, such as the Fc receptor on monocytes (1) or, in the case of hybrid Abs, to structures on target cells (2, 3).

Anti-CD3 mAbs, being bivalent, can also crosslink CD3 molecules on the same T cell and, in this way, deliver a signal. Although this signal usually does not lead to the full display of T cell effector potential, the triggering effect of bivalent anti-CD3 Abs can be measured by assessing a rise in the intracellular Ca^{2+} or by a down-regulation of the CD3/TCR complex itself (4, 5).

In the present study we asked whether the monovalent binding of a ligand to the CD3/TCR complex would be able to transduce a triggering signal to the T cells. As a stable monovalent ligand we used a hybrid mAb in which one binding site is directed to the CD3 and the other to a tumor-associated antigen present on ovarian carcinoma (OVCA) cells. In the absence of OVCA cells this Ab will bind monovalently to CD3, but will not be able to either crosslink or bridge the CD3/TCR complex. Here we show that in the absence of tumor cells this Ab does not transduce triggering signals to the T cells as measured by various criteria. This property makes this Ab a suitable reagent to arm in vitro CTL that subsequently retain their specific effector function for extended periods of time.

Materials and Methods

Antibodies. mAbs were purified over protein A-Sepharose (Pharmacia Fine Chemicals, Uppsala, Sweden) from supernatants of hybridomas that have been described before (6). The Ig produced by the hybrid hybridoma MOV 18-TR 66 was dialyzed against an acetate buffer (25 mM; pH 5.6) and fractionated on a Mono S column (HR 5/5; Pharmacia Fine Chemicals) by elution with a linear salt gradient (0–100 mM NaCl).

T Cells. Resting peripheral blood T cells were purified from buffy coats and further tionated on Percoll gradients (Pharmacia Fine Chemicals) as described (7). The high T cell fraction was further depleted of non-T cells by treatment with a cocktail of rabbit complement (7). The cells were >99% $CD2^+$, >95% $CD3^+$, and negative and Leu-M3 markers. Alloreactive CTL clones were isolated and maintained

T Cell Proliferation. 10^5 T cells were cultured in the presence or absence and Co.

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radiated (6,000 rad) ovarian carcinoma cells (OVCA 432; provided by Dr. R. Knapp, Dana Farber Cancer Institute, Boston, MA) $r 3 \times 10^4$ irradiated peripheral blood monocytes in 200 μl of Iscove's modified Dulbecco's medium supplemented with 5% FCS in 96-well flat-bottomed microplates. Proliferation was measured after 72 h by pulsing with [^{3}H]thymidine (Amersham International, Amersham, UK; 9.25 kBq/well; 185 MBq/mmol sp act).

Determination of Ca^{2+} Mobilization. T cells were loaded with Indo-1-AM (Calbiochem-Behring Corp., La Jolla, CA) as previously described (8), incubated on ice with saturating concentrations of monovalent or bivalent anti-CD3 Abs, and washed with ice-cold PBS. The cells were resuspended into PBS (2 mM Ca^{2+} at 37°C) and changes in Indo-1 fluorescence, as a measure of the cytosolic Ca^{2+} concentration, were recorded with a FACS 440 as described (8). This protocol of prebinding the anti-CD3 Ab was chosen because it minimizes the effect of differences in Ab avidities. In some experiments a rabbit anti-mouse antiserum (Dakopatts, Glostrup, Denmark) was added to the PBS to crosslink the prebound anti-CD3 Ab.

Retargeting Cytotoxic T Cells. 5×10^3 ^{51}Cr-labeled target cells were incubated with effector cells at different E/T ratios in the presence or absence of various mAbs. Specific ^{51}Cr release was measured after 4 h. In some experiments the CTL were pulsed with different concentrations of hybrid anti-CD3/anti-OVCA Ab on ice, washed, recultured at 37°C, and tested at different times for their capacity to kill OVCA cells.

Results

Purification and Characterization of a Bispecific Anti-CD3/anti-OVCA Ab. The Abs produced by the hybrid hybridoma MOV18-TR66 (a fusion product of an IgG1 anti-CD3 and an IgG1 anti-OVCA hybridoma; reference 6) were purified over protein A-Sepharose and fractionated on a Mono S column. Fig. 1 shows that the separation procedure yields three distinct protein peaks that represent the three possible H chain combinations (9).

The hybrid anti-CD3/anti-OVCA Ab can be detected at the level of 1 ng/ml by its capacity to retarget CTL against the tumor cells. This activity was detected only at the beginning of the middle peak (fractions 25–32).

The bivalent anti-CD3 Ab can be detected by its capacity to bridge a CTL to other CD3$^+$ cells, resulting in the reciprocal killing of the T cells (6). This activity was present in the third protein peak and was absent in fractions <34, indicating that the bispecific anti-CD3/anti-OVCA Ab was separated from the bivalent anti-CD3.

Binding of a Monovalent Anti-CD3/Anti-OVCA Ab to T Cells Does not Induce Increase in Cytosolic Ca^{2+} or IL-2 Responsiveness. We tested whether the anti-CD3/anti-OVCA Ab was able to trigger T cells in the absence of OVCA cells. As an early triggering event we measured intracellular Ca^{2+} mobilization, an event that can be detected

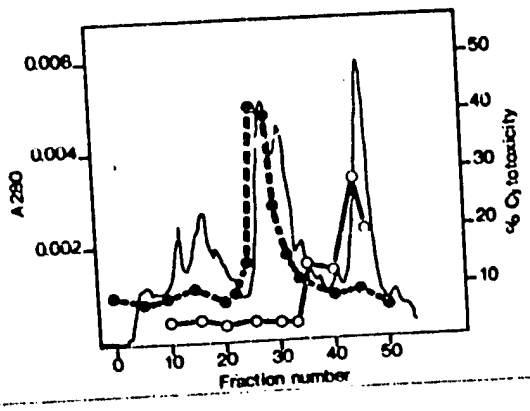

FIGURE 1. Separation of the hybrid anti-CD3/anti-OVCA from the bivalent anti-CD3. Protein A-purified Igs produced by the hybrid hybridoma were separated on a Mono S column. A280 (———); percent of cytotoxicity on OVCA 432 cells obtained by retargeting a nonspecific CTL clone with a 1:2,500 dilution of each fraction (●); reciprocal killing of the CTL in the presence of a 1:100 dilution of the fraction (○).

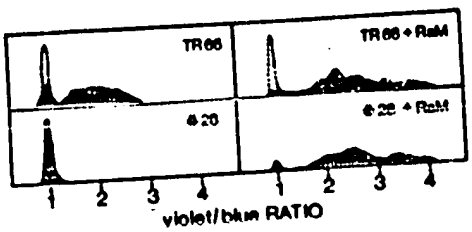

FIGURE 2. Monovalent anti-CD3 does not induce Ca^{2+} mobilization in T cells. T cells were incubated at 0°C with saturating amounts of either bivalent anti-CD3 Ab (TR 66) or with anti-CD3/anti-OVCA Ab (fraction no. 28 of the ion exchange purification), washed, and resuspended in PBS, 2 mM Ca^{2+} at 37°C in the absence or in the presence of a second crosslinking rabbit anti-mouse Ab. Cells were analyzed directly using a FACS. Histograms show the Indo-1 violet/blue ratio, proportional to the cytosolic Ca^{2+} concentration. Dotted line, cells without Ab.

within minutes after receptor perturbation. Fig. 2 shows that when T cells are incubated with saturating amounts of either bivalent anti-CD3 or monovalent anti-CD3/anti-OVCA Ab, only the bivalent anti-CD3 is able to induce Ca^{2+} mobilization, while the monovalent Ab is not effective at all, in spite of the fact that more (1.5 times) Ab has bound to the cells (see below). Moreover, both Abs are equally effective at inducing Ca^{2+} mobilization when crosslinked by a second rabbit anti-mouse Ab, indicating that the inability of the hybrid Ab to trigger is only due to its monovalency.

We next tested whether the monovalent anti-CD3 could induce IL-2 responsiveness in resting T cells. Table I shows that, while the bivalent anti-CD3 can induce proliferation in the presence of exogenous IL-2, the monovalent anti-CD3 is ineffective. In contrast, proliferation did occur after addition of OVCA cells or monocytes, i.e., in conditions that lead to the bridging of the CD3/TCR complex to another cell via the Fc receptor or via the OVCA-specific antigen.

Monovalent Anti-CD3 Does not Induce Downregulation of the CD3 Complex. Bivalent anti-CD3 Abs are known to downregulate the CD3/TCR complex, resulting in the inhibition of T cell function (5). We asked whether the ligation of the CD3 by a monovalent ligand would have a similar effect. T cells were incubated with bivalent or monovalent anti-CD3 Abs at saturating concentrations at 37°C and the Ab present on the cell surface was detected at different times by staining with a goat anti-mouse

TABLE I

Hybrid Anti-CD3/Anti-OVCA Abs Activate T Cells when Bridged to other Cells, but Fail to Induce IL-2 Responsiveness when Bound Monovalently

Cells in culture	[^{3}H]Thymidine incorporated in the presence of:		
	Medium	Anti-CD3	Anti-CD3/anti-OVCA
	cpm		
PBT	50	50	NT
PBT + Mϕ	50	21,450	28,500
PBT + OVCA432	600	900	6,750
PBT + IL-2	2,100	10,600	1,800

PBT were cultured with anti-CD3 Abs (20 ng/ml) or with anti-CD3/anti-OVCA (500 ng/ml) in the presence of accessory cells or 100 U/ml IL-2. Thymidine incorporation was measured after 72 h. Comparable results were obtained in experiments using concentrations of hybrid Ab ranging from 50 ng/ml to 2 μg/ml.

FITC. Table II, Exp. 1, shows that incubation with the bivalent anti-CD3 induced a >90% downregulation of CD3 in 24 h, while the monovalent anti-CD3/anti-OVCA Ab, although binding at 1.5 times the level, did not induce any downregulation of CD3. Similar results were obtained when the T cells were incubated with the hybrid Ab, washed, and recultured at 37°C (Table II, Exp. 2). In this case, the Ab on the cell surface decreased with time, but the total number of CD3 molecules, detected by restaining with anti-CD3, remained unaffected.

Monovalent Anti-CD3/Anti-OVCA Abs Can Be Used to Arm T Cells that Retain Antitumor Activity for Prolonged Periods of Time. The previous results show that purified hybrid Abs in the absence of OVCA cells fail to trigger T cells or induce downregulation of the CD3/TCR complex. These data suggest that this Ab might be a very suitable reagent to confer antitumor activity to T cells that would then maintain this activity until the encounter with the target cell. To exploit this property we incubated a CTL clone with different subsaturating concentrations of anti-CD3/anti-OVCA Ab, then washed and recultured the cells at 37°C, and tested these "armed" cells after different periods of time for their capacity to kill OVCA cells. Fig. 3 shows that armed CTLs efficiently kill OVCA cells, even when a small number of receptors are occupied by the Ab (see for comparison the data in Table II). Near plateau values of killing were obtained with only 3% of receptors occupied and substantial killing was still observed when the number of receptors occupied was too low to detect by immunofluorescence. Furthermore, in spite of the loss of Ab from the membrane, the CTL retained their specific lytic capacity for prolonged periods, and even 2 d after pulsing, cells armed with 100 ng/ml of monovalent Ab still exerted maximal lytic capacity towards the tumor cell, while no CTL activity against other targets, such as EBV transformed B cells or T cell blasts, was displayed (data not shown).

TABLE II

Monovalent Anti-CD3 Fails to Induce Downregulation of the CD3 Complex

Exp.	Antibody	Linear fluorescence values at: 0 h*	24 h*
1	Anti-CD3 5 μg/ml	100	8 (7)
	Anti-CD3/anti-OVCA 10 μg/ml	147	143 (ND)
2	Anti-CD3/anti-OVCA 500 ng/ml	57	4 (107)
	100 ng/ml	19	2 (98)
	10 ng/ml	3	0.7 (103)
	1 ng/ml	0.3	0.5 (104)

T cells (clone AK15) were incubated with Ab at 0°C for 30 min in culture medium. After this, cells were either directly put at 37°C and cultured in the presence of Ab (Exp. 1) or washed three times and cultured in the absence of free Ab (Exp 2). At the times indicated, the cells were washed and stained with a goat anti-mouse FITC Ab. Table values are linear fluorescence values, normalized to 100 for cells stained with bivalent anti-CD3. The values in parentheses are a measure of the total surface CD3, determined by reincubation of the cells with saturating concentrations of bivalent anti-CD3 followed by staining with goat anti-mouse FITC Ab.

* Time of incubation at 37°C.

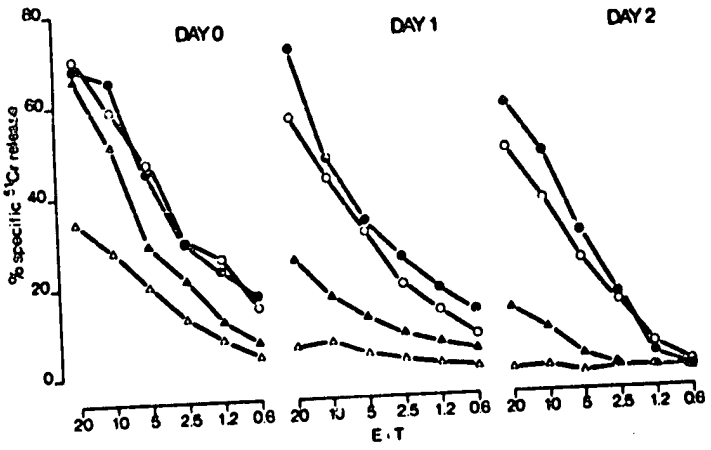

FIGURE 3. CTL armed with hybrid Abs maintain the capacity to kill tumor cells for extended periods of time. CTL clone AK15 (CD8+ anti-HLA Cw3) was pulsed with 500 ng/ml (●), 100 ng/ml (○), 10 ng/ml (▲), and 1 ng/ml (△) of hybrid Ab, washed, and recultured at 37°C. At different times the cells were tested for their capacity to kill OVCA 432 targets.

Discussion

Our results demonstrate that the monovalent ligation of the CD3 complex does not lead to measurable triggering signals in T cells. Using a purified hybrid anti-CD3/anti-OVCA Ab, we show that the binding of this monovalent ligand to T cells does not lead to: (*a*) Ca^{2+} mobilization; (*b*) IL-2 responsiveness; and (*c*) downregulation of the CD3/TCR complex. The lack of stimulatory capacity of this Ab is due to its monovalency, since the same Ab is capable of triggering Ca^{2+} mobilization when crosslinked by a second Ab and, when bridged to OVCA cells, it stimulates proliferation and cytotoxicity. These data, therefore, indicate that crosslinking or bridging are required for signal transduction in T cells.

Our results differ from those of Oettgen et al. (4), who reported that anti-CD3 Fab' fragments prepared from OKT3 can trigger Ca^{2+} mobilization in a T cell tumor line. We do not have a simple explanation for this discrepancy. One possibility is that the tumor T cells might have a special behavior or that the Abs used in that study have a different effect because they recognize a different epitope.

We have exploited the lack of triggering capacity of this hybrid Ab to arm CTL in vitro for specific killing. This strategy, originally suggested by Perez et al. (10) and by Staerz and Bevan (11), requires that the Ab used for arming should not lead to activation and reciprocal killing of the CTL or to downregulation of the TCR. We show here that the use of a monovalent anti-CD3 Ab meets both criteria. First, reciprocal T cell killing (6) can be avoided by removing the bivalent anti-CD3. Killing of FcR+ targets can be avoided by the use of $F(ab)_2$ fragments (6), which are equally effective in retargeting a CTL. Second, specific CTL capacity is retained until the encounter with the target cell, because monovalent anti-CD3 does not lead to capping (12) or downregulation of the CD3/TCR complex.

It is remarkable that a CTL can be armed for killing of OVCA cells by pulsing with a subsaturating concentration of hybrid Ab. Apparently, specific recognition can occur when the fraction of CD3 molecules with hybrid Ab bound is at least 10 times lower than the minimum level of molecules detectable by immunofluorescence, i.e., with a margin of error of the order of 100 molecules per cell.

Summary

We used a purified bispecific antibody (Ab) against CD3 and an ovarian carcinoma (OVCA) antigen to ask whether the binding of a monovalent ligand to CD3 can induce triggering of T cells. In the presence of OVCA cells, this Ab bridges the CD3 complex to the target cell and triggers proliferation and cytotoxicity in T cells. In the absence of target cells, however, this monovalent Ab, even when bound to T cells at high levels, fails to induce any increase in cytosolic Ca^{2+}, nor does it induce responsiveness to IL-2 or modulation of the CD3 complex.

Because it is inert when bound monovalently, this hybrid Ab can be used to arm in vitro CTL cloner, which then retain the capacity to kill the specific tumor for up to 2 d.

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